Expression and effects of social defeat on histamine receptor expression in wild-type and *hdc* $^{-/-}$ zebrafish gut and brain

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Abstract:

Histamine receptors are known to be expressed throughout the peripheral nervous system and are involved in regulating the gut and immune system. The gut-brain axis, which consists of bidirectional signaling between the central nervous system and gastrointestinal tract, links gut functions to emotional and cognitive controls in the brain. Many animal models are known to express histamine receptors in their gut and brain tissue which can be altered by a compromised gut-brain axis like stress. Histamine receptors also play an important role in many gastric and intestinal disorders. The precise expression pattern of histamine receptors in zebrafish gut tissue is unknown, as is whether their expression levels also change with stress. Here, I show that zebrafish gut contains several histamine receptors, but their role involving stress within the gut remains unknown. I found that histamine receptors *hrh1* and *hrh3* as well as the enzyme that synthesizes histamine, histidine decarboxylase (*hdc*), are expressed in zebrafish gut and brain in wildtype and *hdc* knockout adult zebrafish using *in situ* hybridization. Stress induction on wildtype male zebrafish through chronic social defeat and analysis of histamine receptor and *hdc* mRNA levels using quantitative real time PCR showed no differences in subordinate, dominate, or control fish. However, it did provide quantitative data that *hrh1, hrh2,* and *hdc* mRNA expresses in the adult gut. My results demonstrate the first data to suggest histamine receptors are expressed in zebrafish gut, and that even though stress can alter the gut-brain axis, it may not do so through the regulation of these receptors.

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List of Abbreviations

BCIP - 5-bromo-4-chloro-3-indolyl phosphate

DIG - Digoxigenin

HDC - Histidine decarboxylase

HM - Hybridization mix

Hrh – Histamine receptor

ISH – *In situ* hybridization

MUC2 – Mucin glycoproteins

NBT - Nitro blue tetrazolium

PBS - Phosphate-Buffered Saline

PBST - Phosphate-Buffered Saline/Tween

PFA - Paraformaldehyde

qRT-PCR – Quantitative real time polymerase chain reaction

SSCT - Saline-sodium citrate/tween

Tph – Tryptophan hydroxylase

WT – Wildtype

Zlct – Zebrafish lactase enzyme

1. Introduction

Histamine is an endogenous biogenic amine with important roles in the periphery, especially with the regulation of the gut and immune system (Panula et. al., 2015). Histamine modulates its affects through four types of G protein-coupled histamine receptors, *hrh1*, *hrh2*, *hrh3*, and *hrh4*. *Hrh1* receptors are involved with smooth muscle contraction and inflammatory responses (Jutel et al., 2001). *Hrh2* receptors also regulate immune system responses (Jutel et al., 2001) along with gastric acid secretion (Kobayashi et al., 2000). *Hrh3* receptor acts as both an autoreceptor (Arrang et al., 1983) and heteroreceptor (Schlicker et al., 1988), and *hrh3* knockout mice have increased severity of neuroinflammation (Teuscher et al., 2007). *Hrh4* receptors function in mast cells and eosinophils to induce chemotaxis and immune responses (Hofstra et al., 2003**).** Histidine decarboxylase (*hdc*) is the rate-limiting enzyme that synthesizes histamine. Most peripheral histamine is stored in mast cells, lymphocytes, and basophil leukocytes, and it is shown to be stored in enterochromaffin (ECL) and mast cells in the gastric mucosa of cartilaginous and bony fish, amphibians, birds, and mammals (Håkanson et al., 1986). In the mammalian and zebrafish brain, *hdc* neurons are in the tuberomammillary nucleus (TMN) of the posterior hypothalamus (Panula et al., 1984; Watanabe et al., 1984; Eriksson et al., 1998). The zebrafish has been a useful and versatile experimental model since the 1980s to research vertebrate development, genetics, neuronal development, and disease mechanisms, and it is a useful model for studying the histaminergic system (Eriksson et al., 1998).

The bidirectional communication system of the gut-brain axis integrates the central and enteric nervous system to combine gut functions and cognitive centers. Communication occurs through afferent and efferent neural projection pathways, neuroendocrine signaling, and immune activation. Model organisms like rats, guinea pigs, and rabbits are known to express *hrh1*, *hrh2*, and *hrh3* receptors in their gastrointestinal tract with *hrh1* receptors mediating contractile effects of the stomach smooth muscle, *hrh2* receptors mediating relaxing effects of the stomach smooth muscle, and *hrh3* receptors inhibiting neuronal-mediated contraction (Ercan & Turker, 1977; Coruzzi et al., 1991; Grandi et al., 2008). In other peripheral zebrafish tissues, *hrh1* receptor mRNA is detected in intestine, liver, and spleen whereas with *hrh2* and *hrh3* receptor mRNA is detected in the heart and spleen with weaker detection also seen in the liver with *hrh3* receptor RNA (Peitsaro et al., 2007). Furthermore, histamine and its receptors play a role in multiple gastric and intestinal disorders like gastric ulcers, irritable bowel syndrome (IBS), and inflammatory bowel disease

(IBD) where *hrh1* antagonists reduced symptoms in patients with IBS (Wouters et al., 2016), and *hrh1*, *hrh2*, and *hrh4* receptor expression was increased in patients with IBD inflamed GI mucosa (Smolinska et al., 2016). Interestingly, the human GI tract is not shown to express *hrh3* receptors, but patients with food allergy and IBS had elevated levels of mRNA for *hrh1* and *hrh2* receptors (Sander et al., 2006). Developing symptoms of IBS is also speculated to be involved with an irregular gut-brain axis in which mast cells play an important role (Fichna & Storr, 2012), and studies show that mucosal mast cells are augmented in IBS patient's large intestine (Barbara et al., 2007; O'Sullivan et al., 2000).

Currently, there is no other published data on if and where the zebrafish gut express *hrh1*, *hrh2*, and *hrh3* receptors or *hdc*. Due to the teleost-specific genome duplication, one *hrh1* (chromosome 8), two *hrh2* (chromosome 15), and three *hrh3* (chromosome 7) receptor subunits were found in the zebrafish genome, but zebrafish do not have an orthologous *hrh4* gene (Peitsaro et al., 2007). Although zebrafish do not have a stomach, their intestinal development, organization, and function is highly homologous with mammalian intestine (Lickwar et al., 2017) making this model worthwhile to study its gut in relation to the histaminergic system.

Stress, like social situations, can activate inflammatory systems through the neuroendocrine axis (Bierhaus et al., 2003). Short term exposure to stress can alter the microbiota in an animal (Galley et al., 2014), and altered gut microbiota can influence stress responsiveness (Carabotti et al., 2015). Chronic social stress is a stress inducing model that uses a larger or more aggressive animal to induce stress in a smaller animal. This method produces anxiety and social-avoidance behaviors in rodents (Rygula, et al., 2005; Rygula et al., 2006; Golden et al., 2011) and zebrafish (Pavlidis et al., 2011). Zebrafish, like other social animals, establish dominate-subordinate relationships in pairs, and when male zebrafish were manipulated to fight, both the winner and loser experienced changes in their brain transcriptome (Oliveira et al., 2016). Male dominate zebrafish in a 5 day social stress condition were also shown to upregulate the mRNA levels of *hdc* supporting histamines role in stress (Pavlidis et al., 2011). Social defeat also affects the gut-brain axis, as psychological stress alters fecal microbiota and has been shown to downregulate genes involved in the immune response of the terminal ileum in mice (Aoki-Yoshida et al., 2016). Because zebrafish display social hierarchy, and social stress is known to alter *hdc* levels, it is likely that social stress could further alter histamine receptor expression in the brain and gut.

To understand if *hrh1*, *hrh2*, *hrh3*, and *hdc* express in zebrafish gut and how they each express in the adult brain, I used *in situ* hybridization (ISH) on Turku wildtype (WT) and *hdc*

knockout (*hdc* $^{-/-}$*)* adult zebrafish which lack histamine synthesizing activity. If histamine receptors are expressed in the gut, it is important to understand if a lack of endogenous histamine affects histamine receptor expression throughout the gut-brain axis. Social defeat stress was used on adult Turku WT zebrafish, and real-time PCR (qRT-PCR) was used to determine if stress altered histamine receptor expression on the gut and brain. Here, I show that zebrafish gut does express *hrh1, hrh2, hrh3* and *hdc* in WT and *hdc* $^{-/-}$ fish, but the receptors are not altered by the current social defeat stress model.

2. Aims of the Study

The present study addresses two questions: if the zebrafish gut expresses *hrh1*, *hrh2*, *hrh3* and *hdc*, and if social defeat stress will alter these receptors and enzyme mRNA levels in the gut and brain. Specifically, this study aims to: 1. Create a workable ISH method for the zebrafish gut, 2. Characterize *hrh1*, *hrh2*, *hrh3* and *hdc* mRNA signal in the adult gut and brain using ISH and qRT-PCR, and 3. To study if stress affects mRNA levels of *hrh1*, *hrh2*, *hrh3* and *hdc* in the adult gut and brain.

3. Methods

3.1 Experimental Animals

Zebrafish (D. *rerio*) of both sexes were obtained from two different lines, wild-type Turku and *hdc* $^{-/-}$. The Turku WT line has been maintained for over two decades in our lab (Kaslin & Panula, 2001; Chen et al., 2016). Dr. Olov Andersson at Karolinska University established the *hdc* mutant fish, and these mutants have been used in previous research in our lab (Chalas, P., 2020). To establish the *hdc* $^{-/-}$ fish, *hdc* heterozygous mutant fish were bred together to achieve both *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish.

Adult fish were kept at 28°C with a light/dark cycle of 14:10 (lights on at 8:00 A.M.) where they were fed daily once with flake food and twice with live artemia. Fish were housed in continuously cycling Aquatic Habitats Systems with complete exchange of water occurring every 6-10 minutes. Foam filters and activated charcoal filtered the UV-sterilized circulating water. Water temperature (28 ± 0.5°C), pH value (7.4 ± 0.2), and conductivity (450 ± 10µS) was continuously monitored.

The permits to carry out these experiments were obtained from the Office of the Regional Government of Southern Finland, in agreement with the ethical guidelines of the European convention.

3.2 Gut and Brain Dissection

Zebrafish were sacrificed in a 1:10 tricaine (MilliporeSignma, Burlington, MA, USA) dilution for ten minutes. Fish were fasted for 48 h prior to death. The fish were pinned on agarose gel and the abdominal cavity was cut from the gill to the pelvic fin to expose the internal organs. The gut was cut at the start of the intestinal tract towards the mouth and at the anus and then transferred to Phosphate-Buffered Saline (pH 7.4) (PBS) buffer where other organs and fat tissue were removed. The intestinal bulb was cut open roughly until the midline for all *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish. The intestinal bulb was not cut open for Turku WT fish. All waste from the gut was removed with tweezers or squeezed out gently. To remove the brain, the eyes were removed, and the optic nerve was severed. The skull was removed, and the spinal cord was severed at the base of the hindbrain to pull out the brain. All tissues were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA) for two days at 4°C and then dehydrated in a graded 100% methanol and PBS series and stored in 100% methanol at 20°C until further use.

3.3 *In situ* hybridization

Antisense and sense digoxigenin (DIG)-labeled RNA probes were generated using the digoxigenin RNA labeled kit (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. *In situ* hybridization was performed according to the protocol of Thisse and Thisse (2008) with several modifications to improve hybridization efficiency. Fish of both sexes were used. Briefly, gut tissue was digested with proteinase k (Roche Diagnostics, Basel, Switzerland) for 30 minutes, and brain tissue was digested for 1 minute. Prehybridization and hybridization was conducted at 60°C and for 48 h for all probes. 10% dextran sulfate (Amresco, Solon, Ohio, USA) was added to the probe hybridization mix (HM). The temperature was increased to 67°C when gradually changing the HM to Phosphate-Buffered Saline/Tween x 100 (PBST). Washing in successive dilutions of HM to 2 x saline-sodium citrate/tween (SSCT) was increased to 30 minutes while washing in successive dilutions of 0.2xSSCT to PBST was increased to 15 minutes. The blocking buffer contained 4% of 100% sheep serum (Bio Rad, Hercules, California, USA), and incubation in blocking buffer was for a minimum of 1 hour. Tissue samples sat in the anti-DIG antibody solution (Roche Diagnostics, Basel, Switzerland) for 48 hours and then put in PBST for an additional 48 hours at 4°C. The color staining was achieved with chromogen substrate Nitro-blue tetrazolium (NBT) and 5-Bromo-4-chloro-3′-indolyl-phosphate (BCIP) (Roche Diagnostics, Basel, Switzerland). The gut tissue was stained for 14 days while the brain tissue was stained for 48 to 72 hours in all *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish. The gut tissue was stained for 5 days while the brain tissue was stained for 48 hours in all Turku WT fish. When desired staining was achieved, the samples were washed in PBST and placed into 80% glycerol before being mounted and imaged.

Each condition was replicated three times with one brain and gut per replication for the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish. Each condition was replicated one or two times with one brain and gut per replication for the Turku WT fish.

3.4 ISH Imaging

Tissue samples were mounted in 80% glycerol and between two coverslips. Mounted gut samples were imaged with Leica DM IRB inverted microscope and the Leica DFC480 camera (Leica Microsystems, Wetzlar, Germany) at 20x magnification on the exterior side. Gut tissue was imaged in three sections, anterior, middle, and posterior. These sections were visually split up with no formal measurements according to the sections outlined in Wallace et al., 2004. Mounted brain samples were imaged with a Leica DMi1 inverted microscope (Leica Microsystems, Wetzlar, Germany) at 5x and 10x magnification on the dorsal side expect for brains treated with the *hdc* probe which were then imaged with the Leica DM IRB

inverted microscope and Leica DFC480 camera (Leica Microsystems, Wetzlar, Germany) on the ventral side at 5x and 10x magnification.

3.5 Social Defeat Behavioral Experiment

A large approximately 2-year-old male fish was placed in the test tank to allow for tank conditioning for 24 hours. A smaller male fish approximately 1-year old was placed in the test tank with the aggressor fish for ten min the following morning. After 10-min of interaction, a transparent divider was placed in the test tank separating the fish for 24 hours. The smaller fish was then swapped to a new test tank with a new aggressor. The divider was removed for another 10-min interaction. The smaller fish was swapped sequentially to a new aggressor tank every day for four days. Control fish, also approximately 1-year old, were the siblings of the stressed fish and were kept on the same shelf as the aggressor tanks. Control fish did not move tanks. All fish used in this experiment were male Turku WT fish. Following the four-day experiment, the large, small, and control fish were sacrificed in 1:10 tricaine solution, where their gut and brain were removed according to previous description. Tissue samples were flash frozen in nitrogen and stored at -20°C until RNA extraction. The social defeat experiment was repeated twice with 6 fish per category for the first trial, and 8 fish per category for the second trial. The conflict time was increased to 15 min for the second trial.

3.6 RNA Isolation and cDNA Synthesis

RNA was isolated from 14 male 2-year-old dominate fish, 14 male 1-year-old stressed fish, and 14 male 1-year old control fish. RNA was isolated by Qiagen RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. cDNA was synthesized using 2 μg of total RNA and reversed transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and with random hexamer primers (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

3.7 Quantitative RT-PCR

qRT-PCR was performed using a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) and the Lightcycler480 SYBR Green I master mix (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Primers for amplification are listed in table 1. Rpl13a was used as the reference control. Cycling parameters were as follows: 95°C for 5 min, 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. All reactions were duplicated with six to eight biological replicates. Using Ct rpl13a values, data were calculated with the comparative method, and changes of relative gene expression were normalized to rpl13a.

Gene	5-forward	5-reverse	Note
rpl13a	AGAGAAAGCGCATGGTTGTCC	GCCTGGTACTTCCAGCCAACTT	qRT-PCR
hdc	TTCATGCGTCCTCTCCTGC	CCCCAGGCATGATGATGTTC	qRT-PCR/ISH
hrh1	CGAGAAGGAGAGTTTGGCGT	AGGGATTGAGCGTGGAGTTG	qRT-PCR/ISH
hrh2a	GGCCACTAGGGGCGCACTTC	AGCGGAGCAGTGACCGCAAA	qRT-PCR/ISH
hrh2b	GGAACAGGTGCGGCGTATTC	GTGGCTTTATGTTCCCGTGC	qRT-PCR/ISH
hrh3a	CGCCACCGTCCTTGGGAACG	GGGGATGCAAAACCCGCCGA	qRT-PCR/ISH
hrh3b	ACTTCCTCGTGGGTGCTTTCTG	TCCCAGCATCCATTTCCCTGTC	qRT-PCR/ISH
hrh3c	CCATCTCAGACTTCCTTGTTGGAG	AAGTAGTCCATCACCAGCCACAG	qRT-PCR/ISH
tph1a	CTGCCTGAGGAAAGCGAGAT	CATACATCAGCACGCGGTTC	qRT-PCR
tph1b	TACACTCCTGAACCGGACAC	CCCGAGAGAAGCCAACCCTA	qRT-PCR
tph2	GGGCTGTGCAAACAAGATGG	CTCCTGGTAGCACGTGGTTT	qRT-PCR

Table 1. List of primers used in this study for qRT-PCR.

3.8 Statistical Analysis

Quantitative results were analyzed in GraphPad Prism (GraphPad Software, San Diego, CA, USA) using one-way ANOVA test.

4. Results

4.1 *In Situ* Hybridization of Gut and Brain

ISH was used to visualize all histamine receptors and *hdc* in the zebrafish adult gut and brain. The zebrafish lactase (*zlct*) enzyme was also tested due to lactases long known expression and function in mammals' gut (Büller et al., 1990).

The ISH method was optimized using Turku WT fish for the gut whereas the probes and this method was already known to work on the brain. Six out of the eight probes were tested on 1-3 fish for optimization in the gut, but only *zlct*, *hdc*, and *hrh1* signal was shown (Table 2). *Zlct* and *hrh1* expression was seen throughout the entire gut while *hdc* expression is only seen in the anterior gut (Fig. 1). *Hrh2b* mRNA signal was seen in the anterior gut, but the samples hybridized with sense probe were stained in a similar pattern. Samples hybridized with *hrh2a* antisense probe did not display signal in any parts of the gut. Hybridization with *hrh3c* antisense probe showed no signal in any part of the gut (Fig.1). Because this method had not previously been tested on zebrafish gut, the positive signal seen for *zlct*, *hdc*, and *hrh1* mRNA in Turku WT fish provided confirmation to continue this method in the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish and to test the other probes not tested in the Turku WT fish.

	Turku WT			*Hdc +/+*			*Hdc -/-*		
	Ant.	Mid.	Post.	Ant.	Mid.	Post.	Ant.	Mid.	Post.
Zlct	+-	++	+-	+++	+++	---	+++	+++	+--
Hdc	+	-	-	+++	+++	++-	+++	+--	+--
Hrh1	+	+	+	+++	+++	+++	+++	+++	+++
Hrh2a	-	-	-	---	---	+--	++-	+--	---
Hrh2b	-	-	-	+--	+--	---	---	---	---
Hrh3a				--	--	--	+-	--	--
Hrh3b				--	--	--	--	--	--
Hrh3c	---	---	---	+++	++-	+--	---	---	---

Table 2. ISH receptor mRNA signal in the gut of each fish per fish line and antisense probe. A (+) sign represents where signal was seen within one fish, while a (–) sign represents where no signal was seen within one fish. All fish were hybridized with the antisense probe. Thus, this table explains how often mRNA signal was replicated per condition. Blank boxes represent where no data exits. Abbreviations: Ant., anterior gut section; mid., middle gut section; post., posterior gut section.

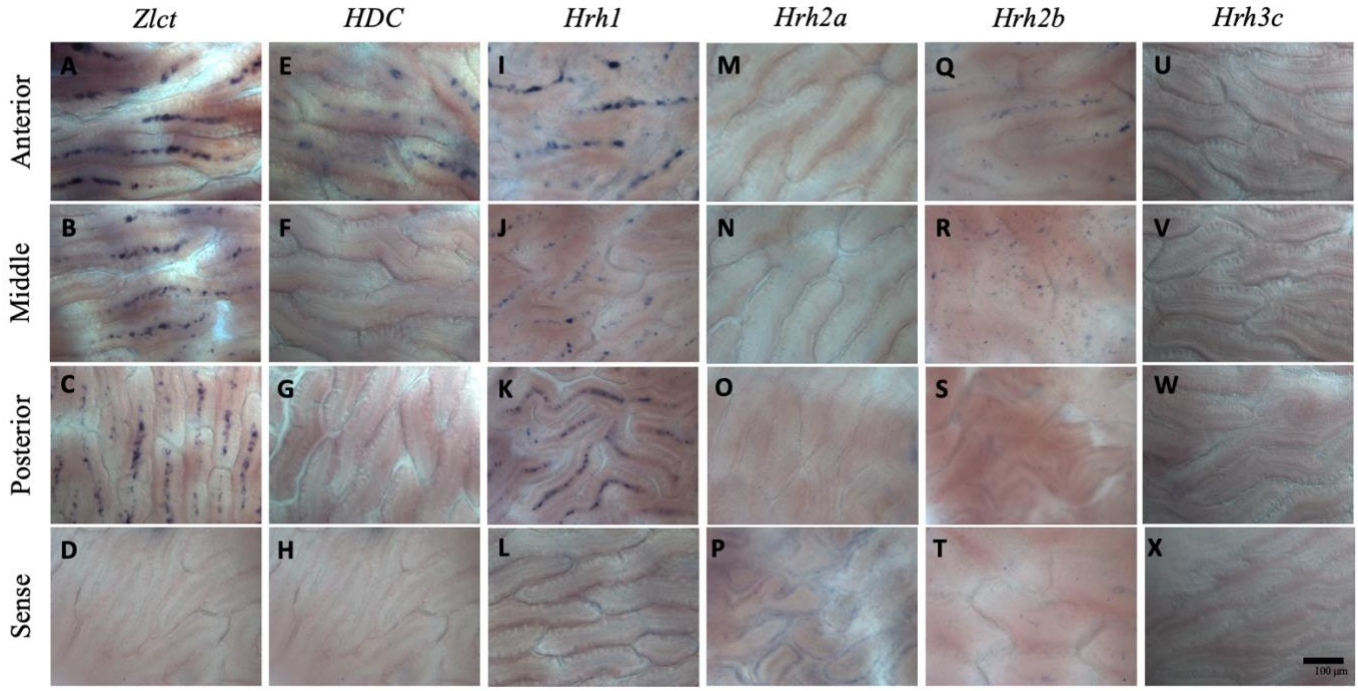

Fig. 1. *In situ* hybridization optimization on Turku wildtype adult zebrafish gut. Representative gut images from the anterior, middle, and posterior sections of the gut with respective probes. The anterior, middle, and posterior gut sections show mRNA signal of the respective antisense probe. The sense probe images are from the anterior gut. Staining is seen in purple. Scale bar is 100 µm.

Similar to the Turku WT fish, *zlct* and *hrh1* mRNA signal was also seen in the *hdc*$^{+/+}$ and *hdc* $^{-/-}$ fish gut (Fig. 2 and 3). There is *zlct* mRNA signal throughout the entire gut for the *hdc* $^{-/-}$ fish but only in the anterior and middle sections of the *hdc* $^{+/+}$ fish (Fig. 2A-F). Out of the three fish tested in each condition, only one *hdc* $^{-/-}$fish showed staining in the posterior section while all three *hdc* $^{+/+}$ fish showed no staining in the posterior gut (Table 2). Staining in the anterior and middle sections of the gut for both fish types also appeared to be more intense than in the Turku WT fish in that the purple staining covered a larger section of the tissue and was darker in color. *Hrh1* mRNA signal was seen throughout the entire gut for both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$fish like that of the Turku WT fish (Fig. 3A-F).

Zlct mRNA signal was not detected in any brain areas. *Hrh1* mRNA signal was seen in the telencephalon and habenula of the brain in both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$fish (Fig. 3 J, K). The staining in the telencephalon appeared to be in the olfactory bulb and the dorsal telencephalon. There was no other staining observed in any other brain areas.

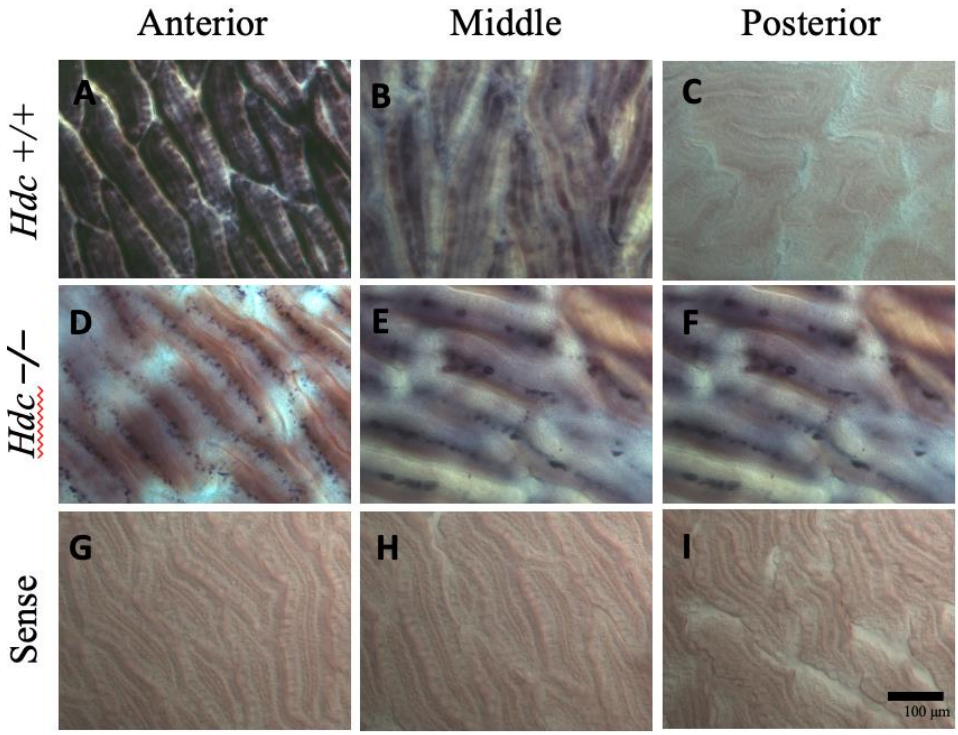

Fig 2. *In situ* hybridization of *zlct* receptor expression in *hdc* knockout gut. A-C, *zlct* antisense mRNA signal in the *hdc* $^{+/+}$ fish where staining is seen in the anterior and middle gut sections. **D-F**, antisense mRNA signal in the *hdc* $^{-/-}$ fish where staining is seen in the anterior, middle, and posterior gut sections. **G-I**, sense probe control images with no mRNA signal. Scale bar is 100 µm.

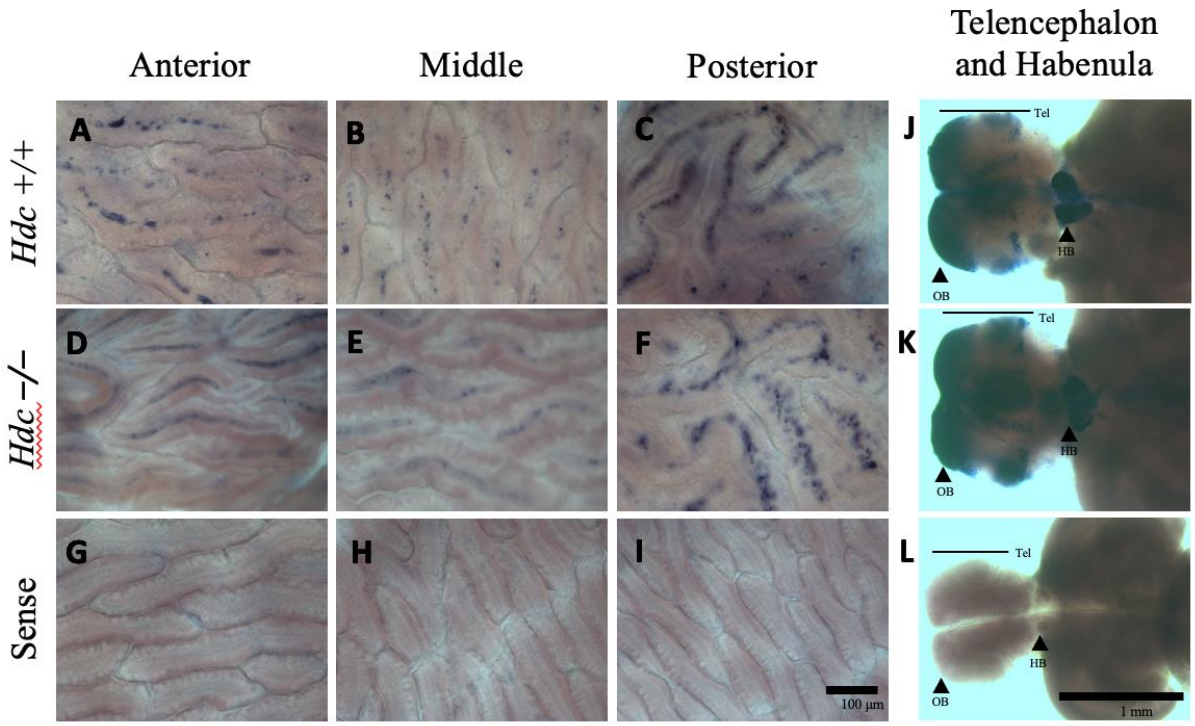

Fig 3. *In situ* hybridization of *hrh1* receptor expression in *hdc* knockout gut and brain. **A-C**, expression of *hrh1* mRNA in the *hdc* $^{+/+}$ fish where staining is seen in the anterior, middle, and posterior gut sections with the antisense probes. **D-F**, expression of *hrh1* mRNA in the *hdc* $^{-/-}$ fish where staining is seen in the anterior, middle, and posterior gut sections with the antisense probes. **G-I**, sense probe control images. **J-K**, antisense mRNA signal in the telencephalon and habenula of the brain of both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish. **L**, sense probe control image with no expression. Scale bars, 100 μm for gut images and 1mm for brain images. Abbreviations: OB, olfactory bulb. HB, habenula. Tel, telencephalon.

Similar to the Turku WT fish, *hdc* mRNA signal was seen in the gut of the *hdc* $^{+/+}$ and *hdc* $^{-/-}$fish, however, staining similar to that in the antisense conditions was seen in the sense probe controls in the anterior and middle gut sections (Fig. 4A-I). *Hdc* mRNA signal was seen in both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish in the ventral hypothalamus (Fig. 4J-M). However, it appears there were less cellular clusters stained in the *hdc* $^{-/-}$ fish compared to the *hdc* $^{+/+}$ fish. When the stained cellular clusters in each group were counted, *hdc* $^{+/+}$ fish had an average of 80 where as *hdc* $^{-/-}$ fish had an average of 62.

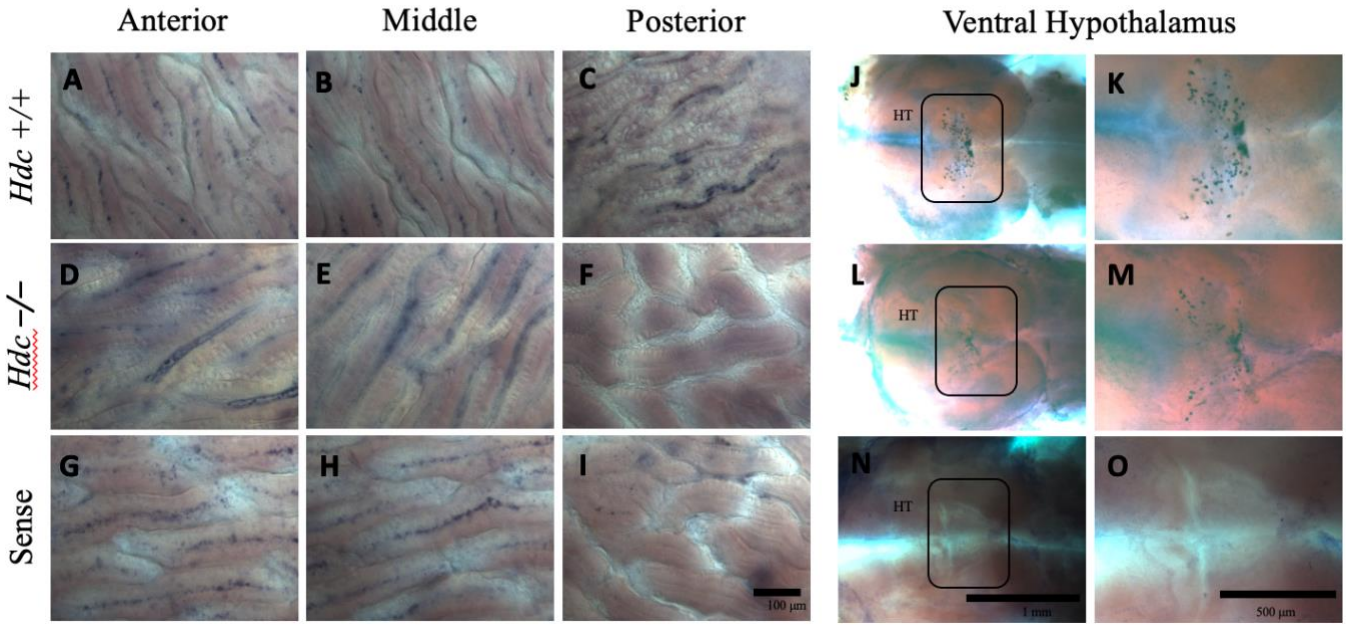

Fig 4. *In situ* hybridization of *hdc* enzyme expression in *hdc* knockout gut and brain. A-C, expression of *hdc* mRNA in the *hdc* $^{+/+}$ fish where staining is seen in the anterior, middle, and posterior gut sections with the antisense probe. **D-F**, expression of *hdc* mRNA in the *hdc* $^{-/-}$ fish where expression is seen in the anterior and middle gut sections with the antisense probe. **G-I**, sense probe control images where staining is seen in anterior, middle, and posterior gut sections. **J-M**, expression of *hdc* mRNA in the ventral hypothalamus of the brain in both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish at 5x magnification with the antisense probe. **K, M**, 10x magnification of their respective counterpart images. **N-O**, sense probe control image with no signal. Scale bars, 100 µm for gut images, 1mm for brain images at 5x magnification, and 500 µm for brain images at 10x magnification. Abbreviations: HT, hypothalamus.

Hrh3a mRNA signal was only seen in the anterior section of the gut of the *hdc* $^{-/-}$ fish (Fig. 5 D). Only two fish were used per category, and only one fish showed *hrh3a* mRNA signal in the anterior gut for the *hdc* $^{-/-}$ fish (Table 2). *Hrh3a* mRNA staining was seen in the dorsal telencephalon and olfactory bulb but not in the habenula (Fig. 5 J, K). No other signal was observed in any other brain areas.

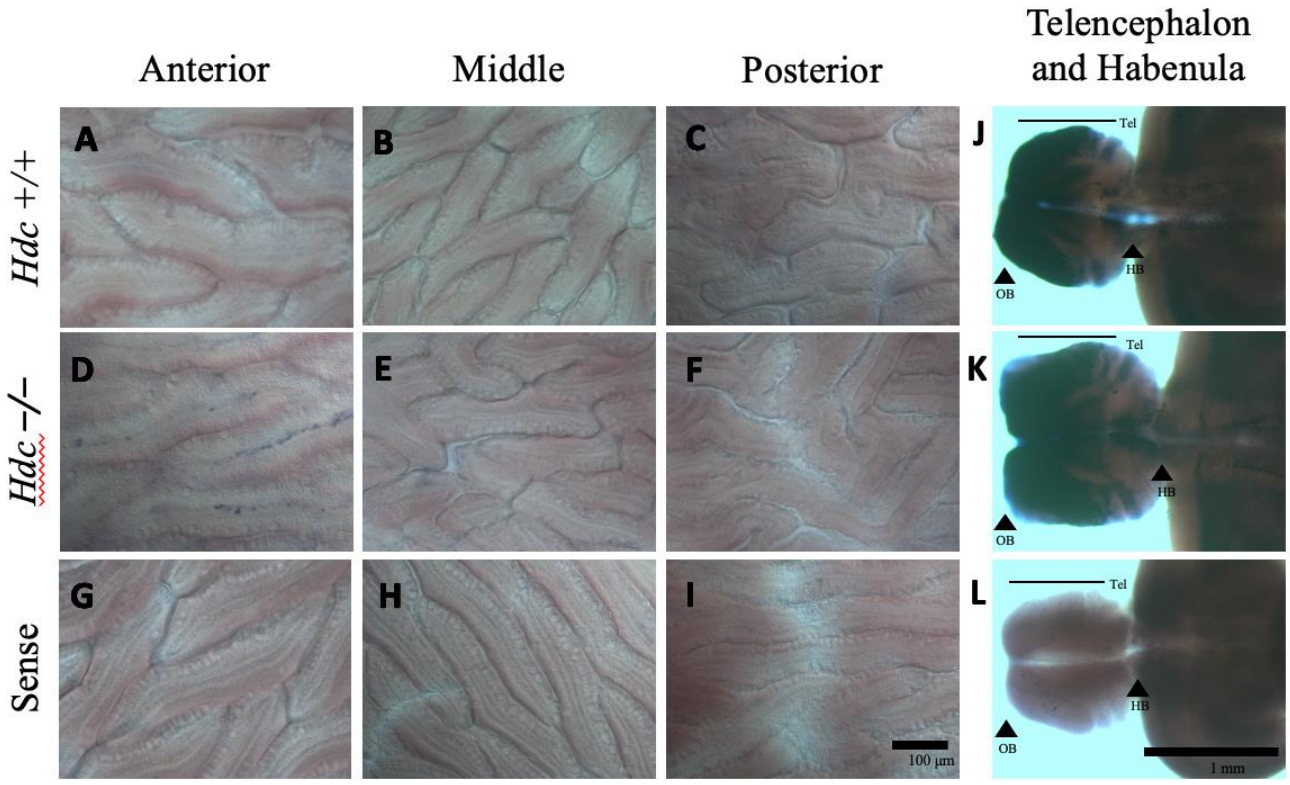

Fig 5. *In situ* hybridization of *hrh3a* receptor expression in *hdc* knockout gut and brain. A-C, expression of *hrh3a* mRNA in the *hdc* $^{+/+}$ fish where no staining is seen the gut with the antisense probe. **D-F**, expression of *hrh3a* mRNA in the *hdc* $^{-/-}$ fish where expression is seen in the anterior gut with the antisense probe. **G-I**, sense probe control images. **J-K**, expression of *hrh3a* mRNA in the telencephalon of the brain of both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish with the antisense probe. **L**, sense probe control image with no expression. Scale bars, 100 µm for gut images and 1mm for brain images. Abbreviations: OB, olfactory bulb. HB, habenula. Tel, telencephalon.

Hrh3b mRNA signal was seen in the habenula of the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ brain (Fig. 6 A, B). Signal appears to be similar between both types of fish. No other signal was seen in any other areas of the brain. Additionally, *hrh3b* signal was not seen in the *hdc* $^{+/+}$ or *hdc* $^{-/-}$ gut.

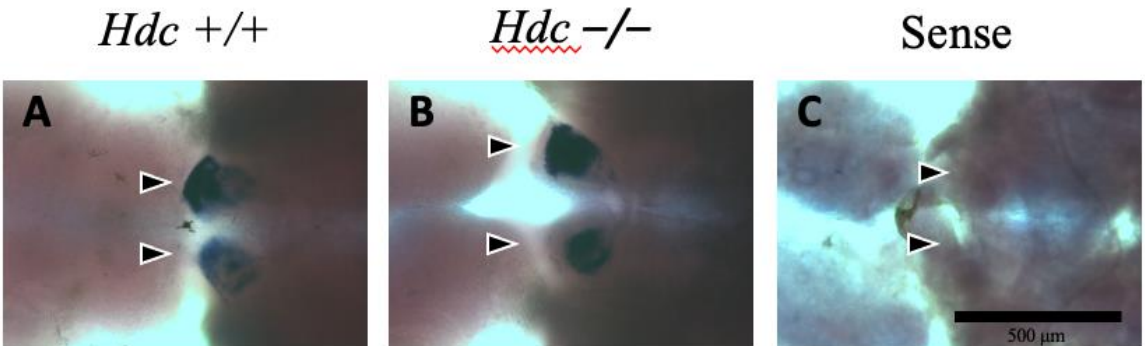

Fig 6. *In situ* hybridization of *hrh3b* receptor expression in *hdc* knockout brain. A, expression of *hrh3b* mRNA in the *hdc* $^{+/+}$ fish with signal in the habenula with the antisense probe. **B**, expression of *hrh3b* mRNA in the *hdc* $^{-/-}$ fish with signal in the habenula with the antisense probe. **C**, sense probe. Scale bar, 500 µm. Arrows point to the habenula.

There was no *hrh3c* mRNA signal detected in the Turku WT fish, but mRNA signal was seen in the *hdc* $^{+/+}$ fish in the anterior and middle gut sections (Fig. 7 A, B). *Hrh3c* mRNA signal was not seen in any of the three *hdc* $^{-/-}$ fish, and only two *hdc* $^{+/+}$ fish middle gut sections were stained, while only one fish's posterior section was stained (Table 2). *Hrh3c* mRNA signal is only seen in the habenula in the brain (Fig. 7 J, K).

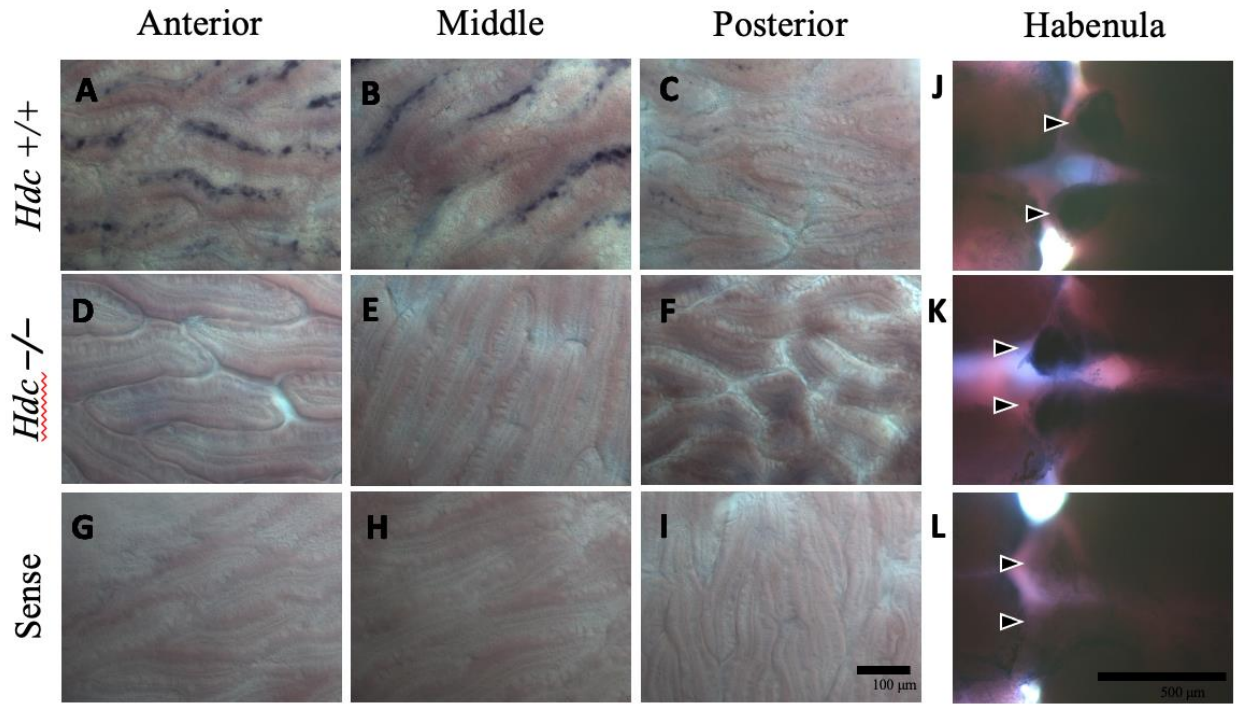

Fig 7. *In situ* hybridization of *hrh3c* receptor expression in *hdc* knockout gut and brain. **A-C**, expression of *hrh3c* mRNA in the *hdc* $^{+/+}$ fish where staining is seen the anterior and middle gut sections with the antisense probe. **D-F**, expression of *hrh3c* in the *hdc* $^{-/-}$ fish where no expression is seen with the antisense probe. **G-I**, sense probe control images where no expression is seen. **J-K**, expression of *hrh3c* mRNA in the habenula of the brain of both the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish with the antisense probe. **L**, sense probe control image with no expression. Scale bar, 100 μm for gut images and 500 μm for brain images. Arrows point to habenula.

Hrh2a, and *hrh2b*, probes were also tested on the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish gut and brain, but no mRNA signal was seen in any areas of the gut or brain.

4.2 Chronic Social Defeat

qRT-PCR analysis was performed to examine transcript levels on relevant genes for the histaminergic system. The three known tryptophan hydroxylase (*tph*) isoforms (*tph1a*, *tph1b*, and *tph2*) were also analyzed due to their known expression in both the brain and gut (Borrelli et al., 2016). No significant differences were measured in different behavioral groups (dominate, subordinate, or control) in either the brain or gut in *hrh1*, *hrh2*, *hrh3*, tp*hrh1*, tp*hrh2*, or *hdc* mRNA expression. Importantly, however, the qRT-PCR analysis revealed that histamine receptors are expressed in the zebrafish gut. *Hrh1*, *hrh2a*, and *hdc* are expressed in both the brain and gut (Fig. 7 A-C, H-J). *Tph1b* and *tph2* are both expressed in the brain and gut, while *tph1a* is only expressed in the brain (Fig. 7 E-G, K-L).

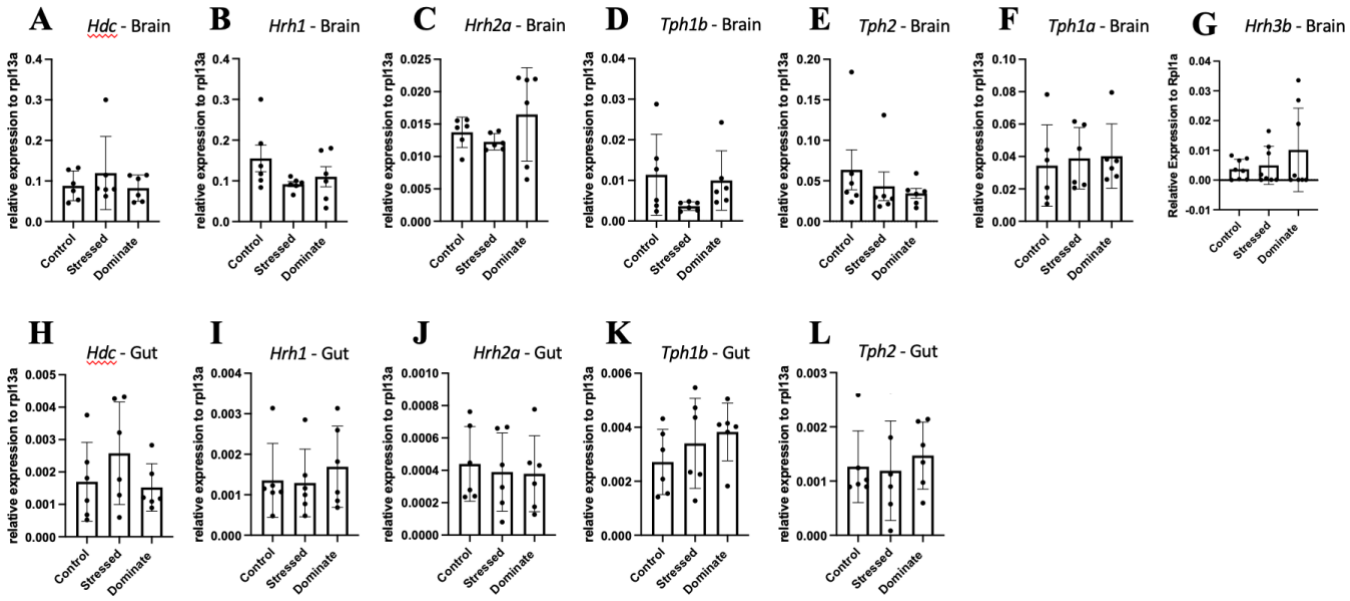

Fig 7. qRT-PCR analysis of social defeat and histamine receptor synthesis in adult Turku WT zebrafish. A-G, Receptor synthesis in the gut. **H-L**, Receptor synthesis in the brain. qRT-PCR analysis relative to expression of the rpl13a gene. n = 6/group.

Hrh2b mRNA expression was significantly reduced in both the gut ($p < 0.01$) and brain ($p < 0.05$) in stressed fish compared with control fish (Fig. 8 A, C). However, this result was not replicated in the second behavioral trial (Fig 8 B, D).

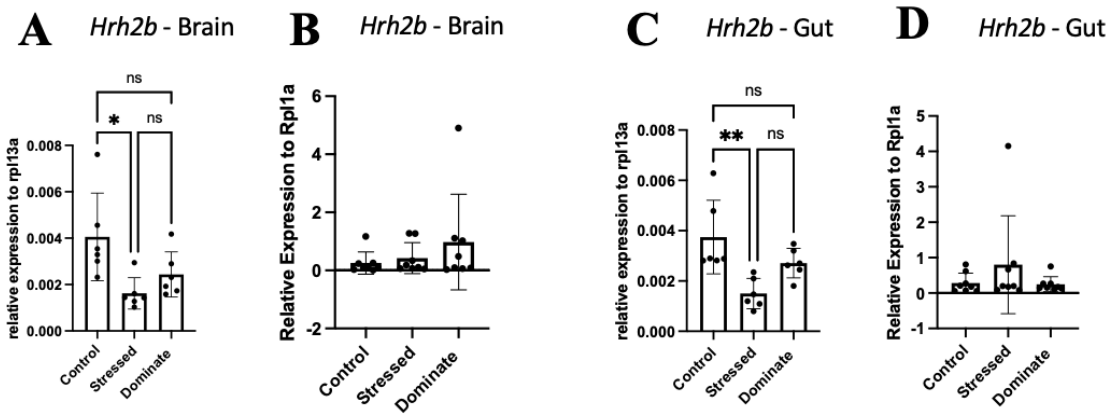

Fig 8. qRT-PCR analysis of social defeat and *hrh2b* receptor synthesis in adult Turku WT zebrafish. A, B, Receptor synthesis in the brain. Significant reduction of *hrh2b* between control and stressed fish in the gut with control fish having higher *hrh2b* mRNA levels than stressed fish (A) (df = 17; p = 0.0044) which was not replicated in a second trial (B). **C, D**, Receptor mRNA levels in the brain. Significant reduction of *hrh2b* between control and stressed fish in the brain with control fish having higher *hrh2b* mRNA levels than stressed fish (C) (df = 17; p = 0.0157) which was not replicated in a second trial (D). qRT-PCR analysis relative to expression of the rpl13a gene. n = 6/group; *p < 0.05; **p < 0.01; one way ANOVA.

5. Discussion

These results show that *zlct*, *hdc*, *hrh1*, *hrh3a*, and *hrh3b* mRNA signal can be visualized in the gut of *hdc* $^{-/-}$ fish, and that *hdc*, *hrh1*, *hrh3*a, *hrh3*b, *hrh3*c mRNA signal can be visualized in the brain of *hdc* $^{-/-}$ fish. There appears to be signal difference in the gut between *hdc* $^{+/+}$ and *hdc* $^{-/-}$ in *hrh3a*, *hrh3c*, and *zlct*. Additionally, the current social stress model did not alter histamine receptor or *hdc* mRNA levels, but it does provide quantitative data that *hdc*, *hrh1*, *hrh2a*, *hrh2b* are expressed in the gut even though the signal was not detected for *hrh2a* or *hrh2b* with ISH. Interestingly, the qRT-PCR data does not show *hrh3*a or *hrh3*c expression in the gut or brain even though these receptors were visualized in the anterior part of the gut and brain using ISH.

Hrh1 mRNA signal was seen in all parts of the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ gut, as well as the Turku WT gut. The qRT-PCR data also confirms the presence of *hrh1* mRNA in the gut. This is in line with previous qRT-PCR research which has detected *hrh1* transcript in the adult zebrafish intestine (Peitsaro et al., 2007), and *HRH1* is known to express in the human GI tract in enterocytes, muscle layer, immune cells, and ganglion cells (Sander et al, 2006). *HRH1* antagonists reduce symptoms of IBS as histamine induced sensitization of the nociceptor transient reporter potential channel V1 (*TRPV1*) via *HRH1* and is known to be the main pathophysiological mechanism underlaying visceral pain in IBS (Wouters et al., 2016). Understanding exactly where *hrh1* mRNA is expressed in the zebrafish gut could lead it to be a useful model in pharmacologically studying and targeting the receptor for future IBS therapies.

Both *hrh2* and *hrh3* are known to express in zebrafish peripheral organs other than the gut (Peitsaro et al., 2007), but there is currently no published data on if they are also expressed in the gut. The current ISH method was unable to show *hrh2* mRNA signal in the gut, but the qRT-PCR data suggests that *hrh2a* and *hrh2b* are expressed there. *Hrh2* ISH has previously been unsuccessful in zebrafish which has been attributed to its low expression level. It may be that *hrh2* mRNA expression level was too low for the ISH method to detect any signal. *HRH2* antagonists have long been used to reduce gastric acid secretion (Black et al., 1972), and IBS patients are reported to have higher levels of *HRH2* mRNA in their GI tract than control patients (Sander et al., 2006). Because *hrh2* are known to have a role in the GI tract, it is likely that the zebrafish gut expresses *hrh2*, and the ISH method will need to be refined to allow for mRNA signal visualization. *Hrh3* have been studied in relation to locomotion in zebrafish (Peitsaro et al., 2007) and aggression (Reichmann et al., 2020), but in

rodents, *hrh3* are shown to be in the GI tract where in rats the receptors are localized to ECL cells and inhibited gut contraction in guinea pigs (Coruzzi et al., 1991; Grandi et al., 2008). The current ISH images only show *hrh3a* mRNA signal in the *hdc* $^{-/-}$ anterior gut in 1 out of 2 fish tested, and *hrh3c* mRNA signal in the *hdc* $^{+/+}$ anterior section in all 3 fish tested and in the middle section in 2 out of 3 fish tested (Table 2). However, *hrh3* qRT-PCR mRNA expression levels were not detected in the gut. Previous qRT-PCR analysis of *hrh3* on larval zebrafish gut has been tested with no expression found (Peitsaro et al., 2007). This suggests that either *hrh3* are not present in the adult gut and the ISH images show false signal, or *hrh3* are present in the adult gut but in low levels which is why ISH images show weak signal in only parts of the gut. Additional ISH and quantitative analysis with a larger sample size should take place before claiming that *hrh3* subtypes are expressed in the adult zebrafish gut.

The zebrafish intestinal architecture is less complex than mammalian counterparts. Mammals have a submucosa and muscularis mucosa while zebrafish do not meaning zebrafish only have a thin connective tissue layer between the epithelium folds and the smooth muscle (Wallace et al., 2004). The zebrafish gut has three epithelial cell types, columnar-shaped absorptive enterocytes, goblet cells, and enteroendocrine cells, and in the layers beneath the epithelium are enteric ganglia (Wallace et al., 2004). Because *HRH1*, *HRH2*, *HRH3*, and *HRH4* mRNAs are found in rat and human goblet cells (Hayashi et al., 2012), exploring zebrafish gut goblet cells would be a promising place to start investigating where these receptors are located. Goblet cells secret mucin glycoproteins (*MUC2*) to coat the GI tract in mucus, and counterstaining could be performed with an *MUC2* antibody to see if histamine receptors express in these cells (Gum et al., 1999). Goblet cells are present in all zebrafish intestinal layers, so epithelial, smooth muscle, and neuronal markers to differentiate between layers should also need to be used. In general, the ISH mRNA signal seen from *hrh1* and *hrh3* is most likely in the epithelium based on previous research of the zebrafish gut differentiating between the gut layers (Wallace et al., 2004; Ng et al., 2005).

Hdc mRNA signal was seen in the anterior section of the Turku WT fish. *Hdc* mRNA signal was seen in the entire gut in the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish, however, the sample hybridized with the sense-oriented probe was similarly stained in the anterior and middle sections. The posterior sample hybridized with the sense-oriented probe was also stained, but the staining pattern appeared to be noise as it was in between the epithelial folds whereas the staining seen with the samples hybridized with the antisense probe appears to be in the epithelial folds (Wallace et al., 2004). Normally, sense probes should produce non-specific binding as they attach to the tissue in a non-complementary manner. However, some genes

are known to be transcribed from the sense and antisense DNA strand meaning some sense probes would produce signal (Katayama et al., 2005; Zhang et al., 2011). Some researchers also mark ISH successful even if the sense condition contains signal if it's reduced and weaker than the antisense signal (Mazzucchelli et al., 1994). Additionally, previous research supports *hdc* expression in the GI tract of other animals, specifically in the ECL and mast cells of the stomach (Nissinen et al., 1992; Håkanson et al., 1986) as histamine is a key stimulant of acid secretion (Andersson et al., 1996). Zebrafish mast cells lack histamine, however (Eriksson et al., 1998). Enteroendocrine cells are mainly located in the anterior section of the zebrafish gut in the epithelium (Wallace et al., 2004) suggesting that the Turku WT ISH images show true *hdc* signal. It may be that the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ gut samples were over stained, and future experiments should reduce the 14-day staining time when using the *hdc* probe. Future experiments could also counterstain with an enteroendocrine cell marker like cytoplasmic pancreatic polypeptide hormone (Langer et al., 1979) to determine if *hdc* is expressed in these cells. Because the qRT-PCR data supports *hdc* expression in the gut in Turku WT fish, and *hdc* is known to have an important role in the GI tract of other animals, it seems likely that the *hdc* ISH signal in the gut is true.

To ensure no false positive ISH results, a positive and negative control was employed. *Zlct* was used as a positive control in the gut because of its long-known role in digesting lactase in milk, and its long-known expression in rodents GI tract (Rings et al., 1992) as well as known peripheral expression in the zebrafish eye (Vihtelic et al., 2005). *Zlct* mRNA signal was seen throughout the entire Turku WT and *hdc* $^{-/-}$ fish, and in the anterior and middle sections of all 3 *hdc* $^{+/+}$ fish. This is the first data to suggest the *zlct* enzyme mRNA occurs in the zebrafish gut. Sense probes were used as negative controls. In general, the sample size was only 2 to 3 fish per fish type and per probe. Due to the novel nature of this research, this small sample size provides evidence to continue exploring receptor expression in the zebrafish gut, but it is too small to claim if expression occurs in the three sections of the gut without additional quantitative evidence. Additionally, the three sections were not strictly measured but were split up based on the outlined sections in Wallace et al, 2004 which may have led to uneven imaging of the gut. The anterior gut section's primary role is nutrient absorption and digestive enzymes are highly present in this region (Wallace et al, 2004). The mid intestine contains enterocytes involved in mucosal immunity which may be analogous to other animal's mucosal mast cells (Wallace et al., 2004). The posterior gut lacks absorptive enterocytes and has been suggested to be analogous with the colon (Wallace et al, 2004; Holmberg et al., 2003). Altogether, the functional differences of the zebrafish gut would

suggest receptor expression could be different in the three sections. A larger sample size and future qRT-PCR performed on all three gut sections instead of the whole gut would lead to more conclusive data about where each receptor or enzyme is located. For example, it is interesting that the entire gut of the Turku WT and *hdc* $^{-/-}$ fish showed *zlct* mRNA signal, but the posterior section of the *hdc* $^{+/+}$ did not. This does not necessarily mean that *hdc* $^{+/+}$ fish do not express *zlct* mRNA in the posterior gut, but rather the dissection or ISH method may have hindered the signal. Future experiments should perform qRT-PCR on the three sections of the gut with *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish to establish if *zlct* mRNA is present throughout or in specific sections of the gut.

Hrh1 mRNA signal has been shown in previous research in the dorsal telencephalon, habenula, locus coeruleus and anterior hypothalamus of larval zebrafish through ISH (Eriksson et al., 1998; Sundvik et al., 2011). My data supports *hrh1* mRNA signal location in the adult dorsal telencephalon and habenula. In zebrafish, *hrh1* are associated with locomotor activity (Rihel et al., 2010), the sleep-wake cycle (Sundvik et al., 2011), and responses to environmental changes (Peitsaro et al., 2007). The zebrafish habenula is associated with learning and prediction (Palumbo et al., 2020). *Hrh2* is known to be expressed in the zebrafish optic tectum, hypothalamus, and locus coeruleus of the brain (Peitsaro et al., 2007). As with previous unsuccessful *hrh2* ISH in the gut, my results were unable to show *hrh2* mRNA signal in the brain, but the qRT-PCR whole brain data shows *hrh2a* and *hrh2b* mRNA being expressed in the brain. *Hrh3* is also known to be expressed in the dorsal telencephalon, posterior hypothalamus, and optic tectum of zebrafish larvae (Sundvik et al., 2011). The current ISH results show *hrh3a* mRNA signal in the telencephalon and habenula, and *hrh3b* and *hrh3c* mRNA signal in only the habenula. No signal was detected in the posterior hypothalamus or optic tectum. *Hdc* mRNA signal has been shown in previous research in the caudal zone of the periventricular hypothalamus (Eriksson et al., 1998), and the current ISH images support that data. *Hdc* positive cells in the zebrafish brain also appear to be where histamine storing neurons are located (Eriksson et al., 1998). In the *hdc* $^{-/-}$ fish, *hdc* signal was still present, but the number of cellular clusters appeared to be fewer than in *hdc* $^{+/+}$ fish. Chen et al (2017) showed that *hdc* $^{-/-}$ fish do not produce histamine even though the cell type is present, and *hdc* $^{-/-}$ express weaker and fewer *hdc* positive cells compared to *hdc* $^{+/-}$ and *hdc* $^{+/+}$ fish. This is also why *hdc* signal is seen in the *hdc* $^{-/-}$ gut. Altogether, this data supports the idea that *hdc* $^{-/-}$ fish have similar histamine receptor expression and *hdc* cells, even though *hdc* is not being produced, compared to WT fish.

The social stress test did not alter histamine receptor mRNA expression in bully/dominate or stressed/subordinate fish. In the first trial, *hrhr2b* mRNA expression was significantly reduced in both the brain and gut of the stressed fish. This result was not replicated in the second trial. In dominate male and female zebrafish, *hrh2* is over expressed in the hypothalamus and is implicated with aggressive behavior, whereas in subordinate male zebrafish, *hrh2* is overexpressed in the telencephalon that innervates the amygdala implicating its role in fear responses (Filby et al., 2010). Because of this, it is surprising that *hrh2* mRNA was not significantly downregulated in stressed fish in the second trial. Previous research shows success in inducing stress and regulating gene expression with social stress in zebrafish. Pavlidis et al (2011) showed dominate and subordinate male zebrafish in a dyadic social stress environment having significantly higher cortisol than control fish, and *hdc* mRNA was upregulated in dominate fish. Furthermore, to investigate social hierarchy in zebrafish offspring, larval F1 generations had lower *hdc* in both dominate and subordinate fish compared to nonhierarchical fish (Sundvik et al., 2021). Thus, it is also surprising that no significant difference of *hdc* mRNA levels was detected in any of the fish in the current results. I used a sample size of only 6 fish in the first trial and 8 fish in the second trial. There was also high variation within groups of the qRT-PCR data, which may result from the dissection method, especially with the gut. All fish were fasted for 48 hours prior to dissection, but there were still high levels of waste in the gut. Removal of waste may have been incomplete and caused some gut tissue to be ripped off and thus not included in the experiment. In the future, this social stress model should be repeated with a larger sample size to account for dissection variation to understand the effect of stress on the histaminergic system in the zebrafish gut-brain axis.

The major limitation of this study was not performing qRT-PCR on the *hdc* $^{+/+}$ and *hdc* $^{-/-}$ fish. The social defeat experiment provided a general idea of which histamine receptors were present in the gut using Turku WT fish, but it would be beneficial to have additional quantitative data to substantiate the ISH findings. Additionally, future experiments should focus on performing ISH and qRT-PCR on the three sections of the gut separately to see if expression levels are consistent or altered throughout the gut. Future experiments should also research what cell type expresses each histamine receptor.

In conclusion, considering that the gastrointestinal tract represents the largest immune organ in the body, it is of particular relevance to study it in regard to how it influences disease and its treatment in relation to the brain. These results, though novel, suggest a

starting point for future experiments targeting the histaminergic system in relation to immune responses in the gut-brain axis.

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